PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 15/00, 15/12, C12N 9/10
C12N 15/00, 15/54, 15/62

A1

(11) International Publication Number: WO 93/19091

(43) International Publication Date: 30 September 1993 (30.09.93)

(21) International Application Number: PCT/AU93/00105

(22) International Filing Date: 17 March 1993 (17.03.93)

(30) Priority data:
PL 1411 18 March 1992 (18.03.92) AU

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: TRIPARTITE FUSION PROTEINS OF GLUTATHIONE S-TRANSFERASE

(57) Abstract

A tripartite fusion protein comprises a first amino acid sequence corresponding to a glutathione-S-transferase enzyme at its COOH terminus with a second amino acid sequence corresponding to a binding entity, particularly an immunological binding entity, followed by a third amino acide sequence corresponding to a different polypeptide fused with said binding entity. The protein may include a cleavable link between the first and second amino acid sequences and/or the second and third amino acid sequences. Recombinant DNA molecules and expression vectors coding for these fusion proteins are also disclosed.

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TRIPARTITE FUSION PROTEINS OF GLUTATHIONE S-TRANSFERASE

This invention relates to the expression in bacteria such as *Escherichia coli* of foreign polypeptides as fusions with glutathione-S-transferase (GST).

In International Patent Application No. PCT/AU88/00164, the disclosure of which is included herein by reference, it is disclosed that a fusion protein having a foreign polypeptide component fused to the enzyme glutathione-S-transferase (E.C. 2.5.1.18), preferably to the carboxy-terminal of the enzyme, avoids several of the difficulties associated with known fusion proteins, for instance fusions wherein the foreign polypeptide is expressed as a fusion with *E.coli* β-galactosidase, in that the GST fusion proteins are generally soluble and can be purified from bacterial lysates under non-denaturing conditions, for example by affinity chromatography on a column of immobilised glutathione. The GST enzyme in the fusion protein may be derived from the parasite helminth *Schistosoma japonicum,* or it may be derived from other species including humans and other mammals.

The GST fusion proteins disclosed in International Patent Application No. PCT/AU88/00164 may be used as such, since the foreign polypeptide component thereof often retains its antigenicity and functional activity. Alternatively, the fusion protein may be cleaved to provide the foreign polypeptide as a synthesis product, and when the production of such a synthetic polypeptide is desired a cleavable link may be provided in the fusion protein between the glutathione-S-transferase component and the foreign polypeptide component. The cleavable link is preferably one which can be cleaved by a site-specific protease such as thrombin, blood coagulation Factor Xa, or the like.

Thus, in one aspect this earlier application discloses a fusion protein comprising a first amino acid sequence corresponding to the enzyme glutathione-S-transferase and, preferably fused to the COOH-terminus thereof, a second amino acid sequence corresponding to a foreign polypeptide (and optionally a cleavable link between these two components), as well as recombinant DNA molecules, expression vectors, and host cells for use in the production of such a fusion protein.

In another aspect, this earlier application discloses an expression vector (such as a bacterial plasmid) for use in the production of a foreign polypeptide, wherein the vector has inserted therein a nucleotide sequence capable of being expressed as the glutathione-S-transferase enzyme followed by at least one restriction endonuclease recognition site for insertion of a nucleotide sequence capable of being expressed as a foreign polypeptide fused with the COOH-terminus of the glutathione-S-transferase enzyme, optionally with a sequence capable of being expressed as a cleavable link between the enzyme and the foreign polypeptide.

Preferably, expression of the fusion proteins by the expression vectors is under the control of the *tac* promoter which enables inducible, high-level production of these fusion proteins. Preferably also, the expression vectors contain the *lac* Iq gene, so that they can be used in any *E.coli* strain.

Polypeptides expressed in *E.coli* as fusions with GST[1] have proven useful for the analysis of protein-DNA and protein-protein interactions. Part of the reason for this is that, in contrast to many other expression systems, the purification of GST fusion proteins involves non-denaturing conditions so that the expressed polypeptide is recovered in a relatively native state and retains at least some of its normal properties. Examples include GST fusions with GCN4[2] and PEA3[3] that behave at site-specific DNA binding proteins with properties similar to those of their normal counterparts, while fusions with the retinoblastoma gene product (Rb)[4,5], Adenovirus E1A[6], c-Kit[7] or the TATA binding protein (TBP)[8]

retain specific interactions with other proteins. In addition, GST fusion proteins have been used to identify and characterise previously unknown properties of polypeptides. Thus, nucleotide sequences that are recognised by myogenin homomers[9], MyoD[10] and c-Myc[11] have been delineated through Polymerase Chain Reaction (PCR) amplification of DNA selected by GST-fusion proteins from mixtures containing random sequences, while fusions with TBP[12], Rb[4,13] and c-Myc[6] have been used to characterise previously unknown interactions with other proteins. Such approaches have been extended recently by the use of biotinylated GST-c-Abl fusions to detect interactions with proteins separated on Western blots[14] and the identification of a protein that interacts with c-Myc through screening of a CDNA library with a radiolabelled GST fusion[15].

In order to simplify the use of GST fusion proteins for such applications, in work leading to the present invention, the GST expression vector pGEX-2T[1] has been modified so that it directs the expression of glutathione-S-transferase (GST) fusion proteins that also contain one or more IgG binding domains from *Staphylococcus aureus* protein A. Such tripartite fusion proteins can be detected directly with commercially available antibody-enzyme conjugates, thus simplifying the use of GST fusion proteins as probes for the analysis of protein-protein interactions. In particular, in these fusion proteins the GST COOH-terminus is followed by one or more of the five naturally occurring IgG binding domains from *Staphylococcus aureus* protein A. Previous studies have shown that the IgG binding property of these domains is retained when all five are expressed as a GST fusion protein[16] or when they are expressed as isolated domains[17,18]. It was an object of this modification to provide a non-radioactive and generalised detection system using a tripartite fusion protein containing GST followed by one or more immunoglobulin binding domains and finally the polypeptide of interest, where the tripartite fusion protein would also bind to IgG. Such a fusion protein could be purified on glutathione-agarose beads, used as a probe for protein-DNA or protein-protein interactions, and detected using standard antibody-enzyme conjugates.

The present invention provides a fusion protein comprising a first amino acid sequence corresponding to a glutathione-S-transferase enzyme fused at its COOH terminus with a second amino acid sequence corresponding to a binding entity, followed by a third amino acid sequence corresponding to a different polypeptide fused with said binding entity.

In accordance with a preferred embodiment of the present invention, there is provided a fusion protein comprising a first amino acid sequence corresponding to glutathione-S-transferase enzyme fused at its COOH terminus with a second amino acid sequence corresponding to at least one immunoglobulin binding entity such as an immunoglobulin binding protein or one or more binding domains thereof, followed by a third amino acid sequence corresponding to a different polypeptide fused with said immunoglobulin binding entity.

In another aspect, the present invention also provides a recombinant DNA molecule comprising a nucleotide sequence which codes on expression for a fusion protein in which a binding entity is fused with the COOH-terminus of a glutathione-S-transferase enzyme, followed by a different polypeptide fused with said binding entity.

A preferred embodiment of this aspect of the present invention provides a recombinant DNA molecule comprising a nucleotide sequence which codes on expression for a fusion protein in which at least one immunoglobulin binding entity such as an immunoglobulin binding protein or one or more binding domains thereof is fused with the COOH-terminus of a glutathione-S-transferase enzyme, followed by a different polypeptide fused with said immunoglobulin binding entity.

If desired, the fusion protein may also include a cleavable link, for example a link which can be cleaved by a site specific protease such as thrombin or blood coagulation Factor Xa. Such a cleavable link is preferably included between the GST moiety and the binding entity; however, a cleavable link may additionally or

alternatively be included between the binding entity and the polypeptide fused therewith.

The present invention also provides expression vectors and host cells having inserted therein a recombinant DNA molecule in accordance with this invention, as well as methods of producing the fusion protein of this invention using such expression vectors and host cells as disclosed in detail in prior International Patent Application No. PCT/AU88/00164, the disclosure of which is incorporated by reference.

In another aspect, the present invention also provides an expression vector having inserted therein a nucleotide sequence capable of being expressed as a glutathione-S-transferase enzyme followed by a binding entity fused with the COOH-terminus of said glutathione-S-transferase, and at least one restriction endonuclease recognition site for insertion of a further nucleotide sequence capable of being expressed as a different polypeptide fused with said binding entity. Such an expression vector may, if desired, also include a nucleotide sequence capable of being expressed as a cleavable link as discussed above.

In a preferred embodiment of this aspect, the present invention also provides an expression vector having inserted therein a nucleotide sequence capable of being expressed as a glutathione-S-transferase enzyme followed by at least one immunoglobulin binding entity such as an immunoglobulin binding protein or one or more binding domains thereof fused with the COOH-terminus of said glutathione-S-transferase, and at least one restriction endonuclease recognition site for insertion of a further nucleotide sequence capable of being expressed as a different polypeptide fused with said immunoglobulin binding entity. Optionally, this expression vector may also include a nucleotide sequence capable of being expressed as a cleavable link as discussed above.

The GST enzyme in the fusion protein may be derived from *Schistosoma japonicum*, or it may be derived from other species including humans and other mammals.

The binding entity which is included within the fusion protein in accordance with the present invention may be any member of a specific binding pair, including for example an antigen/specific antibody binding pair (especially an antigen/specific monoclonal antibody binding pair), and avidin/biotin binding pair, or preferably an immunoglobulin binding protein/immunoglobulin binding pair.

The immunoglobulin binding entity included in preferred fusion proteins in accordance with this invention may be any protein having immunoglobulin binding affinity, more particularly binding affinity for immunoglobulin G (IgG). Alternatively, one or more immunoglobulin binding domains of such a protein may be incorporated in the fusion protein. A particularly preferred binding entity comprises *Staphylococcus aureus* protein A or one or more of the IgG binding domains thereof, however the invention also encompasses the use of other immunoglobulin binding proteins such as Protein G, Protein GG and chimaeric Protein A/G. It will be appreciated that the immunoglobulin binding protein or binding domain(s) may be selected so as to optimise the binding to IgG in the intended application. Thus, in view of the limited specificity of protein A and in particular its poor binding to sheep, goat and rat IgG, other immunoglobulin binding entities may be chosen for particular applications. Protein G has a different spectrum of binding (as described by Lew *et.al.*[16]), and the immunoglobulin binding entity may even be a synthetic IgG binding domain as described by Lowenalder *et.al.*[19].

The precise nature of the different or "foreign" polypeptide which forms part of the fusion protein of this invention is not essential. Accordingly, the present invention extends to such fusion proteins which incorporate any polypeptide or protein of interest as the different or "foreign" polypeptide. By

way of example, this polypeptide or protein of interest may be a particular antigen, with the resulting fusion protein being useful in a diagnostic test method and kit for detection of specific antibody to the particular antigen in a sample, such as a serum sample, using the well-known enzyme immunoassay (EIA) techniques. In such a diagnostic test using a GST/protein A/antigen fusion protein as the EIA capture phase, specific antibody if present in the sample will bind to both the antigenic portion and the protein A portion of the fusion protein thereby providing a larger surface area on the capture phase for binding of the antibody, leading to greater sensitivity of reaction.

In another example of the use of the fusion proteins of this invention in diagnostic tests, the polypeptide or protein of interest may be a "reporter" entity, particularly an enzyme such as alkaline phosphatase, urease, horseradish peroxidase, or any other enzyme used in colorimetric or chemiluminescent determinations. Such a fusion protein, for example a GST/Protein A/horseradish peroxidase fusion protein, can be used directly in various immunoassay procedures to detect antigen-antibody reactions by binding to the protein A moiety, and then directly detected by standard colorimetric or chemiluminescent methods. In this way, the requirement for various antibody/reporter molecule conjugates (such as goat anti-mouse/HRPO or rabbit anti-human/HRPO conjugates) for use in such immunoassay procedures can be avoided.

It will of course be appreciated that the inclusion of the GST moiety in the fusion protein of the present invention enables the ready purification of the fusion protein by affinity chromatography on immobilised glutathione. In addition, where the fusion protein is to be used as an EIA capture phase, the fusion protein may be immobilised on a solid substrate by first coating the solid substrate with anti-GST antibody and then contacting the coated substrate with the fusion protein.

Further features of the present invention are illustrated, by way of example only, in the following Example and in the accompanying drawings. This Example

illustrates the construction of altered pGEX2T expression vectors that direct the expression of GST fusions containing one, two or three protein A IgG binding domains. These fusion proteins bind to IgG and can be detected using commercially available antibody-enzyme conjugates. Applications of these vectors to the analysis of protein-protein interactions are illustrated by experiments using an NH_2-terminal region of SV40 T antigen as a probe on Western blots of cell extracts, and by binding assays of immobilised GST fusion proteins probed with GST-protein A fusions.

In the drawings:

Figure 1 shows detection of GST-protein A fusion proteins on Western blots. Cells transformed with plasmids directing the expression of GST-protein A fusion proteins containing one (a), two (b, pAGEX2T) or three (c) IgG binding domains were grown in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and proteins analysed by electrophoresis through SDS-polyacrylamide gels followed by transfer to nitrocellulose. Polypeptides containing IgG binding domains were visualised by probing with AP-conjugated rabbit anti-chicken antibodies, followed by detection of enzyme activity *in situ*.

Figure 2 shows the structure of pAGEX2T. Indicated in the drawing are the IPTG-inducible *trp-lac* hybrid promoter (Ptac), the regions encoding the *Schistosoma japonicum* glutathione-S-transferase (GST), a thrombin cleavage site (Th) and two copies of the *S.aureus* protein A IgG binding domain B(PA) followed by unique BamHI, SmaI and EcoRI restriction sites. Also represented are the position of unique PstI and EcoRV restriction sites, the gene encoding β-lactamase (amp^r), an origin of replication (ori), and an over-expressed allele of the lac repressor ($lacI^q$). The nucleotide sequence of a single IgG binding domain and the polypeptide that it encodes are also given.

Figure 3 shows the sensitivity of detection of GST-protein A fusion proteins. Equal quantities of purified GST or GST-protein A (GST-PA) were applied to nitrocellulose from left to right as 5 µl drops in four-fold dilutions from

1 µg to 1 ng. Air dried strips were probed with G53, a monoclonal antibody directed against GST followed by AP-conjugated goat anti-mouse antibodies (Anti-GST), with AP-conjugated rabbit antibodies (AP-Conjugated Rabbit Ab), with either R819, a rabbit antisera raised against Rb (Anti-Rb) or a non-specific rabbit serum (Sigma) (Rabbit Serum) followed by AP-conjugated goat anti-rabbit antibodies, or with Ab419, a monoclonal antibody specific for SV40 T antigen followed by AP-conjugated goat anti-mouse antibodies (Anti-TAg). After washing, enzyme activity was detected *in situ*.

Figure 4 shows the use of GST-protein A-SV40 TAg as a probe for protein-protein interactions on Western blots. A Western blot of a HeLa whole cell extract was probed with GST-protein A(a) or GST-protein A-SV40 TAg (1-272) (b) at a concentration of 25 µg/ml in NT buffer containing 150 mM, 500 mM or 1 M NaCl. After washing in the same buffer containing 150 mM NaCl, bound protein was detected by probing with AP-conjugated rabbit antibodies followed by enzyme detection *in situ*.

Figure 5 shows that Rb is amongst the proteins detected by GST-protein A-SV40 TAg. Western blots of HeLa cells (H) that express Rb and WERI-1 cells (W) that do not, were probed with a rabbit antisera raised against Rb followed by AP-conjugated goat anti-rabbit antibodies (Anti-Rb) or with GST-protein A-SV40 TAg (1-272) followed by a pool of antibodies directed against SV40 TAg and then AP-conjugated goat anti-mouse antibodies (GST-PA-TAg). Enzyme activity was detected *in situ*.

Figure 6 shows the binding of protein A-TEF-1 to GST-SV40 TAg immobilised on beads. Purified GST-Protein A-TEF1 (1-168) was cleaved with thrombin and 1,3 or 10 µl incubated with glutathione-agarose beads carrying GST or GST-TAg (1-260). After washing, probe retained on the beads was analysed by probing Western blots with AP-conjugated rabbit antibodies and detection of enzyme activity *in situ*. Total represents 1.5 µl of probe. The triangles indicate the increasing quantity of probe from left to right.

Figure 7 shows the binding of complex probes to GST fusion proteins on beads. Purified GST-protein A-TBP and GST-protein A-SV40 TAg (1-272) were cleaved with thrombin, mixed together and incubated with glutathione beads carrying GST or GST-SV40 TAg (1-260) and retained probe analysed as above. Total represents 1/25 of the probe added to each sample of beads.

EXAMPLE

MATERIALS AND METHODS

Construction of plasmids.

A single IgG binding domain (domain B) of *S.aureus* protein A[20] was isolated by PCR amplification from the plasmid pRIT 5 (Pharmacia[21]) using the oligonucleotides 5'-TGAGATCTGCGGATAACAAATTCAAC-3' and 5'-ACGGATCCTTTTGGTGCTTGAGCATC-3'. Amplification was for 20 cycles of 94 °C 1 minute, 55 °C for 1½ minutes and 74 °C for 1½ minutes using 25 ng pRIT5 and 100 ng each oligonucleotide in a solution containing 10 mM Tris (HCl) pH 8.3, 1.5 mM $Mgcl_2$, 50 mM KCl, 200 µm dATP, dCTP, dGTP and dTTP and 2.5 units Thermus aquaticus DNA polymerase (Cetus).

The PCR product was incubated for 1 hour at 37 °C with the restriction endonuclease Sau3A in 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl and after electrophoresis through a 2% low-gelling-temperature agarose gel a 185 bp fragment was purified and ligated with BamH1 cleaved and calf intestinal alkaline phosphatase treated pGEX-2T in a 10 µl reaction containing 100 ng of vector, 50 ng purified fragment, 20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP and 1 unit T4 DNA ligase (Pharmacia). After 16 hours at 20 °C the ligation reaction was transformed into competent *E.coli* cells of the strain HB101 and spread onto agar plates containing 50 µg mM ampicillin. Individual colonies appearing after 15 hours growth at 37 °C were screened for their ability to direct expression of an enlarged glutathione-S-transferase by inoculation into 1 ml of L-broth, growth for 4 hours with agitation at 37 °C, and a further 2 hours growth after the addition of isopropylthio-B-D-galactoside (IPTG) to 0.1 mM. Cells were pelleted out of a 50 µl sample, heated at 90 °C for 2

minutes in SDS sample buffer and separated by electrophoresis through a 10% SDS - polyacrylamide gel. Proteins were visualised by staining with Coomassie Brilliant Blue and a clone identified that directs the expression of a 33 kDa polypeptide.

No precipitate was observed in the lane corresponding to cells transformed with pGEX-2T while a faint precipitate corresponding to the 33 kDa GST polypeptide indicated that this molecule contains a functional protein A IgG binding domain. By repeating these cloning steps a plasmid was isolated that expresses a GST fusion protein containing two protein A IgG binding domains which gives much stronger signal on Western blots when probed with the antibody conjugate. This plasmid, called pAGEX-2T (see Figure 2), retains the multiple cloning sites of pGEX-2T in the same frame at the 3′ end of the protein A domains. Repeating these cloning steps again led to a plasmid containing three protein A domains.

Expression of a region of SV40 T antigen encoding amino acids 1-272 as a GST-protein A fusion protein was made possible by PCR amplification of the plasmid pGEX1-SV40 TAg (1-272)[22] with the oligonucleotides 5′-TAGGATCCATGGATAAAGTTTTAAACAG-3′ and 5′-GCTGCATGTGTCAGAGG-3′. The PCR product was incubated with BamHI and EcoRI and inserted into BamHI and EcoRI cleaved pAGEX2T producing the plasmid pAGEX2T-SV40 TAg (1-272). A plasmid encoding the NH_2-terminal 168 amino acids of TEF-1 as a GST-protein A fusion (pGEX2T-TEF1 (1-168)) was produced by inserting a 500 bp BamHI fragment of pGEX2T-TEF1 (1-426) (described below) into the BamHI site of pAGEX2T. Similarly, a 1 kb BamHI-EcoRI fragment of pGEX2T-TBP was inserted into BamHI and EcoRI cleaved pAGEX2T to generate the plasmid pAGEX2T-TBP.

A plasmid capable of expressing entire TEF-1 as a GST fusion protein (pGEX2T-TEF1 (1-426)) was constructed by isolating a 1.3 kb NcoI-BgIII fragment from pXJ40-TEF1A[23], treating with the Klenow fragment of *E.coli*

DNA polymerase I (Klenow) to fill in the ends, followed by litigation with SmaI cleaved pGEX2T. In order to express the first 260 amino acids of SV40 T antigen as a GST fusion protein the single PflMI restriction site in pGEX2T was removed by cleavage and religation after incubation with Klenow. A BamHI-EcoRI fragment from pAGEX2T-SV40-TAg (1-272) was introduced into this vector, and using the unique PflMI restriction site within the SV40 coding sequences, the PflMI-EcoRI fragment was replaced with a PflMI-HindIII fragment of pKT260[24] to produce the plasmid pGEX2T-SV40 TAg (1-260). Standard procedures were followed for manipulations of DNA and *E.coli* transformation. GST fusion proteins were purified from bacterial cultures as described[1].

Western transfer and detection of GST-protein A fusion proteins.

Protein samples were separated by electrophoresis through 10% SDS-polyacrylamide gels and transferred to nitrocellulose at 4 °C in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% v/v methanol. Transfer was at 100 V for 1 hour or at 30 V for 15 hours, after which blots were blocked for 30 minutes at 20 °C in PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4) containing 5% non-fat dried milk powder. After rinsing in PBS, blots were probed for 1 hour at 20 °C with a 1:5,000 dilution in PBS of an alkaline-phosphatase (AP) conjugated rabbit anti-chicken antibody (Chemicon). Blots were then washed three times in PBS with enzyme activity detected by incubation in the dark at 20 °C in a solution containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg/ml nitro blue tetrazolium (Sigma) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

The sensitivity of detection of fusion proteins was assessed by dot blots in which equal quantities of purified GST or GST-protein A were diluted in 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) (NT buffer) containing 1% bovine serum albumin (fraction V, Sigma) (BSA) and applied in four-fold dilutions as 5 µl drops to nitrocellulose strips. After air drying, blots were blocked as above and probed for 1 hour at 20 °C with antisera diluted in NT buffer containing 3% BSA.

Blots were then rinsed three times in NT buffer, probed for 1 hour at 20 °C in NT buffer containing 3% BSA and a 1:5,000 dilution of AP-conjugated goat anti-mouse or anti-rabbit antibodies (Promega), and developed as above.

Probing of Western blots with GST-protein A fusion proteins.

Cell pellets or aliquots of a HeLa whole cell extract[25] were heated to 90 °C in sample buffer, separated through 10% SDS-polyacrylamide gels and transferred to nitrocellulose or Immobilon-P (Millipore). After blocking as above, blots were cut into strips and probed for 2 hours at 20 °C with purified GST-protein A or GST-protein A-SV40 TAg (1-272) at a concentration of 25 µg/ml in NT buffer containing 3% BSA. Blots were washed three times in NT buffer and bound proteins then detected by probing with AP-conjugated rabbit antibodies, or with a pool of monoclonal antibodies raised against T antigen followed by AP-conjugated goat anti-mouse antibodies.

Bead binding assays.

Different GST fusions were purified on glutathione-agarose beads and stored at -20 °C while still bound to the beads in PBS containing 15% glycerol. Beads were diluted with fresh glutathione-agarose beads so as to equalise the concentration of bound protein between samples. Prior to an assay, 25 µl of beads were washed in a microfuge tube with 1 ml of PBS containing 5 mM $MgCl_2$, 0.5% Tween 20 and 0.5% BSA, and collected by brief centrifugation. Thrombin-cleaved GST-protein A fusion proteins were added to the drained beads in 30 µl of the same buffer and incubated for 1 hour at 4 °C with frequent agitation. Beads were then washed twice with 1 ml of buffer without BSA, drained, and the retained protein A fusion analysed by Western blotting as above.

Thrombin cleavage of fusions was carried out while proteins were immobilised on beads (200 µl, containing up to 200 µg fusion protein) in 200 µl buffer containing 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 50 mM NaCitrate, 1 mM $CaCl_2$, 1% BSA and 2 µg human thrombin (Sigma). After incubation at 20 °C for 1 hour beads were pelleted and the supernatant removed. The beads

were washed once with 50 mM Tris-HCl (pH 8.0) and the combined supernatants stored at -80 °C.

RESULTS

Construction of GST-protein A vector pAGEX2T

A single IgG binding domain of *S.aureus* protein A (domain B)[20] was isolated by PCR amplification of DNA of the plasmid pRIT5[21] using oligonucleotide primers specific for domain B and inserted into the BamHI site of pGEX2T[1]. The primers were designed such that the BamHI site was re-formed at a position corresponding to the COOH-terminus of the IgG binding domain so that further domains could be introduced in the same manner. *E.coli* transformants were identified containing plasmids that direct the expression of GST fusion proteins containing one, two or three binding domains. All of these GST-protein A fusion proteins were recognised when Western blots of cell lysates were probed with AP-conjugated rabbit antibodies, and the sensitivity of detection increased with the number of binding domains (Figure 1). Correcting for the different amounts of each fusion protein, the signal increases about 50-fold from one to two domains, and about 2-fold from two to three domains. Since it was desired to minimise the size of the GST-protein A carrier and since adding a third binding domain had a relatively minor effect on the sensitivity of detection, the plasmid that encodes two domains (called pAGEX2T) was chosen for all subsequent work. This vector contains unique BamHI, SmaI and EcoRI restriction sites at a position corresponding to the COOH-terminus of the second IgG binding domain and in the same reading frame as in the parent plasmid pGEX2T, followed by termination codons in all three frames (Figure 2). Fusions expressed using this vector consist of GST followed by a thrombin cleavage site, two protein A IgG binding sites and finally the protein of interest.

Sensitivity of detection of GST-protein A fusion proteins.

The sensitivity with which the GST-protein A fusion encoded by pAGEX2T could be detected was investigated by probing dilutions of purified protein with different antisera (Figure 3). Both GST-protein A and GST alone

were detected by a monoclonal antibody specific for GST (Anti-GST), but GST-protein A detection was more sensitive, presumably because of the additional effect of antibody binding to the IgG binding domains. A similar sensitivity of about 4 ng protein was observed for GST-protein A probed with enzyme-conjugated rabbit antibodies (AP-Conjugated Rabbit Ab) and as expected there was no signal for GST alone. Enhanced sensitivities of less than 1 ng were observed when GST-protein A was probed first with an unconjugated antibody (Rabbit Serum, rabbit Anti-Rb or MAb419, an Anti-TAg monoclonal antibody) followed by the appropriate enzyme-conjugated second antibody. Amplification of signal in these cases may reflect polyvalent binding of enzyme-conjugated secondary antibodies to primary antibodies bound to the protein A domains. Similar sensitivities were observed for tripartite fusion proteins containing GST, protein A and portions of SV40 T antigen or of the transcriptional activator TEF-1. This suggests that the presence of extraneous polypeptide sequences on both flanks of the protein A domains does not hinder the binding of antibodies.

GST-protein A fusions as probes for Western blots

Some applications of the pAGEX2T vector can be illustrated using an NH_2-terminal region of SV40 T antigen (amino acids 1-272) that is known to interact with several different proteins[26,27,28] and when expressed as a GST fusion protein is able to autoregulate and transactivate the SV40 early and late promoters in *in vitro* transcription reactions [22]. A DNA fragment encoding this region was inserted into pAGEX2T resulting in the expression of a tripartite fusion protein of 67 kDa. When this purified fusion protein was used as a probe on Western blots of a transcriptionally active HeLa whole cell extract[25] distinct bands over a range of molecular weights were recognised (Figure 4, 0.15 M, b). Blots incubated with the same probe in solutions containing higher concentrations of NaCl showed different patterns (0.5 M, 1.0 M) while only faint signals were observed on blots probed under the same conditions with GST protein A alone (a). The strength of signal on these blots was reduced with lower concentrations of probe while the pattern obtained was influenced by the type of transfer membrane, the transfer conditions, post-transfer renaturation of proteins[29] and the

type of protein blocker and solute concentrations used during probing (data not shown).

In order to test the possibility that the strong signal at about 110 kDa on blots probed with SV40 T antigen in 150 mM NaCl was the Rb protein, extracts from HeLa cells that express Rb, and WERI-1 cells that have a homozygous deletion of Rb[30], were probed with antibodies specific for Rb or with the GST-protein A-SV40 T antigen fusion protein. When Immobilon-P was used as the transfer membrane in such experiments no difference in the intensity of the 110 kDa band could be detected between the two cell lines (data not shown). However, when proteins were transferred to nitrocellulose, a band was observed in the 110 kDa region that was only present in HeLa cells and that co-migrated with a band detected by anti-Rb antibodies (Figure 5). Nitrocellulose and Immobilon-P have different binding properties and it is presumed that a species present in both cell types interacts with the GST-protein A-SV40 T antigen fusion protein but transfers efficiently only to Immobilon-P where it obscures the interaction with Rb.

These experiments also reveal several other cellular proteins that can interact with SV40 T antigen. These interactions are independent of GST since the same species are recognised on blots probed with fusion protein that had been cleaved with thrombin and consists of the protein A domains fused to the SV40 T antigen region, while distinct patterns were observed on blots probed GST-protein A fusion proteins containing portions of the transcription activators TEF-1, PEA3 or TBP (data not shown).

Binding of cleaved GST-protein A fusion proteins to GST fusion proteins on beads.

Several studies have used GST-fusion proteins absorbed to glutathione-agarose as an affinity matrix to purify molecules from whole cell lysates[4,6,7,14] or from specifically programmed *in vitro* translation reactions[6,12,13]. As an extension to this approach, polypeptides expressed using the pAGEX2T vectors have been

used as probes for interactions with GST fusion proteins immobilised on beads. Such experiments require that the GST portion of the GST-protein A fusion protein is first removed by treatment with thrombin so that the probe does not simply absorb to the glutathione-agarose. After incubation of cleaved probe with beads bearing different GST fusion proteins, the beads are washed and retained probe is analysed by Western blotting followed by detection with enzyme-antibody conjugate.

A probe consisting of the protein A domains fused to the NH_2-terminal 168 amino acids of TEF-1 does not bind to beads bearing GST alone but is retained on beads carrying GST fused to the first 260 amino acids of SV40 T antigen (Figure 6). This interaction is specific since a breakdown fragment of the protein A-TEF-1 fusion does not bind to either type of beads, and the intact fusion fails to bind to beads bearing GST fused to NH_2-terminal or COOH-terminal fragments of TEF-1 or to entire TBP (data not shown). Although both fusions possess DNA binding activities, the interaction does not appear to be mediated by DNA since the binding of probe to beads is not affected by the addition of DNA to 30 μg/ml or of DNAase I to 10 μg/ml.

Another illustration of this method takes advantage of cases where two or more cleaved GST-protein A fusion proteins are of different sizes and so can be used together in binding assays. When cleaved fusion proteins containing protein A fused to TBP or the NH_2-terminal 272 amino acids of SV40 T antigen were mixed and used as a probe, binding to GST-SV40 TAg (1-260) beads above the background seen on GST beads was only observed for protein A-TBP (Figure 7).

DISCUSSION

In this Example, the GST expression vector pGEX2T has been modified in order to simplify the use of GST fusion proteins in the analysis of protein-protein interactions by producing tripartite fusion proteins that contain IgG binding domains. Polypeptides expressed in this system can be purified on glutathione-agarose under non-denaturing conditions and can be detected without further

modification using standard reagents and without the use of radioactive materials. This system is less disruptive and more general than some other methods. Previous studies have used GST fusions as probes for protein-protein interaction after labelling by iodination[15,31] or biotinylation[14]. Such treatments might sometimes obscure interactions because of the incorporation of labelling moieties at binding sites. An alternative non-disruptive method for detecting GST fusion proteins would be to use monoclonal antibodies specific for GST. Although this approach has been used to probe blots with GST-SV40 TAg (1-272) and obtain results similar to those obtained with a GST-protein A fusion, the method requires an additional specific reagent and extra manipulations. Such a detection system would also not allow the detection of cleaved GST fusion proteins as used in the binding experiments described above.

Detection of GST-protein A fusion proteins require either an enzyme-linked antibody that itself binds to protein A, or else antibody that binds to protein A and an appropriate enzyme-linked second antibody. GST-protein A fusions have been detected directly with AP-conjugated rabbit antibodies and indirectly using mouse monoclonal antibodies or rabbit serum followed by enzyme-linked goat anti-mouse or anti-rabbit antibodies. Fusion proteins could also be detected using antibodies from other species such as guinea-pig, cow, human, pig or horse, or might utilise other enzyme-conjugates or include biotin-avidin binding steps so as to further amplify the signal. Antibodies from species such as the goat, sheep, rat and chicken bind poorly to protein A[32] and so could not be used by themselves.

The binding properties of polypeptides may be affected by the presence of GST and IgG binding domains in the fusion proteins. Removal of GST from a tripartite fusion protein containing amino acids 1-272 of SV40 T antigen by cleavage with thrombin did not affect the pattern of bands detected on a Western blot of HeLa cell proteins. In addition, similar patterns were obtained when the same region expressed as a GST fusion protein, but lacking the protein A domains, was used as a probe and detected using a pool of monoclonal antibodies

directed against GST. GST fusion proteins have been observed to mirror the properties of their native counterparts in many instances[2-16,22,31].

Previous immunological studies have identified molecules that interact with amino acids 1-272 of SV40 T antigen including Rb[26], heat shock protein 70 (hsp70)[27] and a protein of 185 kDa[28]. The present experiments indicate that Rb is recognised on Western blots of whole cell lysates by a GST-protein A fusion protein containing this region of SV40 T antigen (Figure 5). Several other proteins are detected on these blots, and these may include hsp70 and the 185 kDa protein as well as other species that may reveal previously unknown interactions. The present experiments also suggest that there are direct interactions between SV40 T antigen and the transcription factors TEF-1 and TBP. The possibility that SV40 T antigen might interact with TEF-1 was previously suggested by experiments indicating that the ability of T antigen to activate SV40 transcription is at least partially dependent on DNA sequences in the SV40 enhancer to which TEF-1 binds[22,33]. Interactions between TBP and other virus-specified transactivators have already been demonstrated for Adenovirus E1A[8], Herpes Simplex virus VP16[34] and cytomegalovirus IE2[12].

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within its spirit and scope. The invention also includes all the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively , and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A fusion protein comprising a first amino acid sequence corresponding to a glutathione-S-transferase enzyme fused at its COOH terminus with a second amino acid sequence corresponding to a binding entity, followed by a third amino acid sequence corresponding to a different polypeptide fused with said binding entity.

2. A fusion protein according to claim 1, wherein said binding entity is a member of a specific binding pair.

3. A fusion protein according to claim 2, wherein said specific binding pair is selected from an antigen/specific antibody binding pair, an avidin/biotin binding pair or an immunoglobulin binding protein/immunoglobulin binding pair.

4. A fusion protein comprising a first amino acid sequence corresponding to a glutathione-S-transferase enzyme fused at its COOH terminus with a second amino acid sequence corresponding to at least one immunoglobulin binding entity, followed by a third amino acid sequence corresponding to a different polypeptide fused with said immunoglobulin binding entity.

5. A fusion protein according to claim 4, wherein said immunoglobulin binding entity is a natural or synthetic IgG binding protein, or one or more IgG binding domains thereof.

6. A fusion protein according to claim 5, wherein said IgG binding protein is *Staphylococcus aureus* protein A or one or more IgG binding domains thereof.

7. A fusion protein according to claim 4, wherein said immunoglobulin binding entity is selected from Protein G, Protein GG or chimaeric Protein A/G.

8. A fusion protein according to any one of claims 1 to 7, further comprising a cleavable link between said first and second amino acid sequences and/or a cleavable link between said second and third amino acid sequences.

9. A fusion protein according to claim 8, wherein said cleavable link is one which can be cleaved by a site specific protease.

10. A fusion protein according to claim 9, wherein said cleavable link is one which is cleavable by thrombin or blood coagulation Factor Xa.

11. A fusion protein according to any one of claims 1 to 10 for use in a diagnostic immunoassay for detection in a sample of one member of an antigen/specific antibody binding pair, wherein said third amino acid sequence comprises all or a binding fragment of the other member of said binding pair.

12. A fusion protein according to any one of claims 1 to 10 for use in a diagnostic immunoassay, wherein said third amino acid sequence comprises a reporter molecule or label.

13. A fusion protein according to claim 12, wherein said reporter molecule or label is an enzyme.

14. A fusion protein according to claim 13, wherein said enzyme is selected from alkaline phosphatase, urease or horseradish peroxidase.

15. A recombinant DNA molecule comprising a nucleotide sequence which codes on expression for a fusion protein according to any one of claims 1 to 14.

16. An expression vector comprising a nucleotide sequence according to claim 15, and operatively linked thereto an expression control sequence for expression of said fusion protein.

17. A host cell transformed with an expression vector according to claim 16.

18. A method of producing a fusion protein which comprises the step of culturing host cells according to claim 17 under conditions such that said fusion protein is expressed in recoverable quantity.

19. A method according to claim 18, comprising the further step of recovering said fusion protein from the cell culture.

20. A method according to claim 19, wherein said fusion protein is recovered by contacting said fusion protein with immobilised glutathione, optionally after first lysing said host cells.

21. A method according to any one of claims 18 to 20, comprising the further step of cleaving said fusion protein at the cleavable link or links therein, if present.

22. An expression vector having inverted therein a nucleotide sequence capable of being expressed as a glutathione-S-transferase enzyme followed by a binding entity fused with the COOH-terminus of said glutathione-S-transferase, and at least one restriction endonuclease recognition site for insertion of a further nucleotide sequence capable of being expressed as a different polypeptide fused with said binding entity.

23. An expression vector according to claim 22, wherein said binding entity is a member of a specific binding pair.

24. An expression vector according to claim 23, wherein said specific binding pair is selected from an antigen/specific antibody binding pair, an avidin/biotin binding pair or an immunoglobulin binding protein/immunoglobulin binding pair.

25. An expression vector according to any one of claims 22 to 24, wherein said nucleotide sequence includes a sequence capable of being expressed as a cleavable link between said enzyme and said binding entity and/or between said binding entity and said different polypeptide.

26. An expression vector having inserted therein a nucleotide sequence capable of being expressed as a glutathione-S-transferase enzyme followed by at least one immunoglobulin binding entity fused with the COOH-terminus of said glutathione-S-transferase, and at least one restriction endonuclease recognition site for insertion of a further nucleotide sequence capable of being expressed as a different polypeptide fused with said immunoglobulin binding entity.

27. An expression vector according to claim 26, wherein said immunoglobulin binding entity is a natural or synthetic IgG binding protein, or one or more IgG binding domains thereof.

28. An expression vector according to claim 27, wherein said IgG binding protein is *Staphylococcus aureus* protein A, or one or more IgG binding domains thereof.

29. An expression vector according to claim 26 wherein said immunoglobulin binding entity is selected from Protein G, Protein GG or chimaeric Protein A/G.

30. An expression vector according to any one of claims 26 to 29, wherein said nucleotide sequence includes a sequence capable of being expressed as a cleavable link between said enzyme and said immunoglobulin binding entity and/or between said immunoglobulin binding entity and said different polypeptide.

31. The GST-protein A expression vector pAGEX2T described herein.

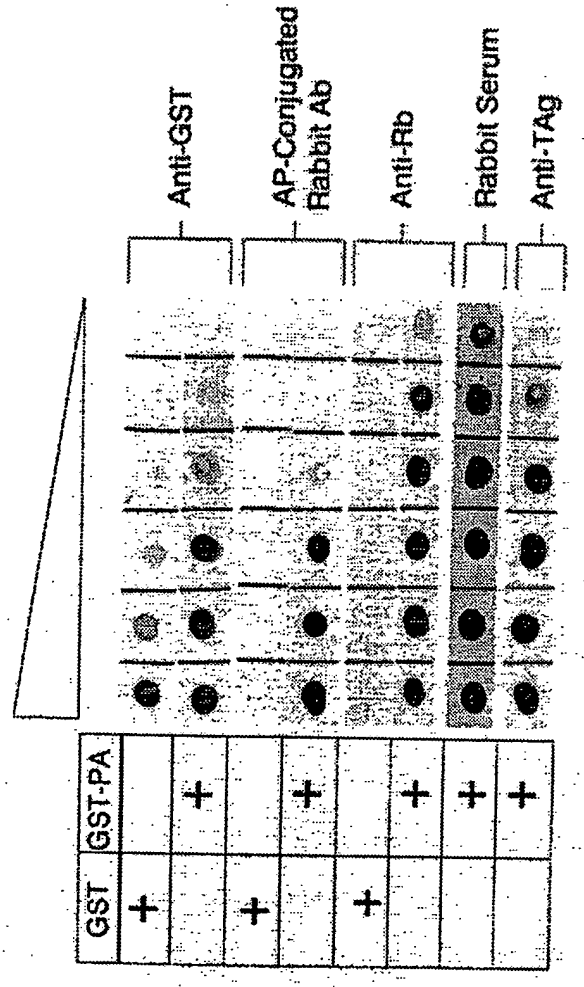

FIG 3

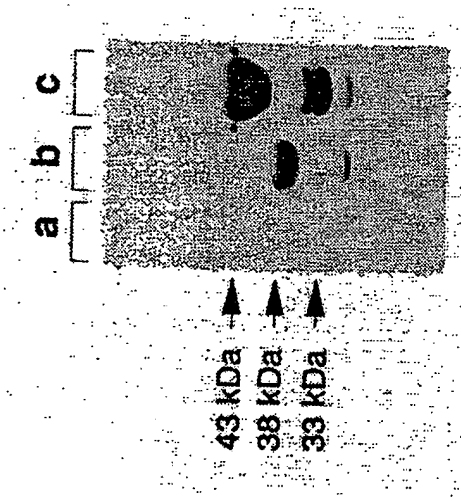

FIG 1

SUBSTITUTE SHEET

Domain B of Protein A

...GCG GAT AAC AAA TTC AAC AAA GAA CAA CAA AAT GCT TTC TAT GAA ATC TTA CAT TTA CCT AAC TTA AAC GAA GAA CAA CGC AAT GGT TTC
Ala Asp Asn Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe

ATC CAA AGC CTA AAA GAT GAC CCA AGC CAA AGC GCT AAC CTT TTA GCA GAA GCT AAA AAG CTA AAT GAT GCT CAA GCA CCA AAA...
Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys

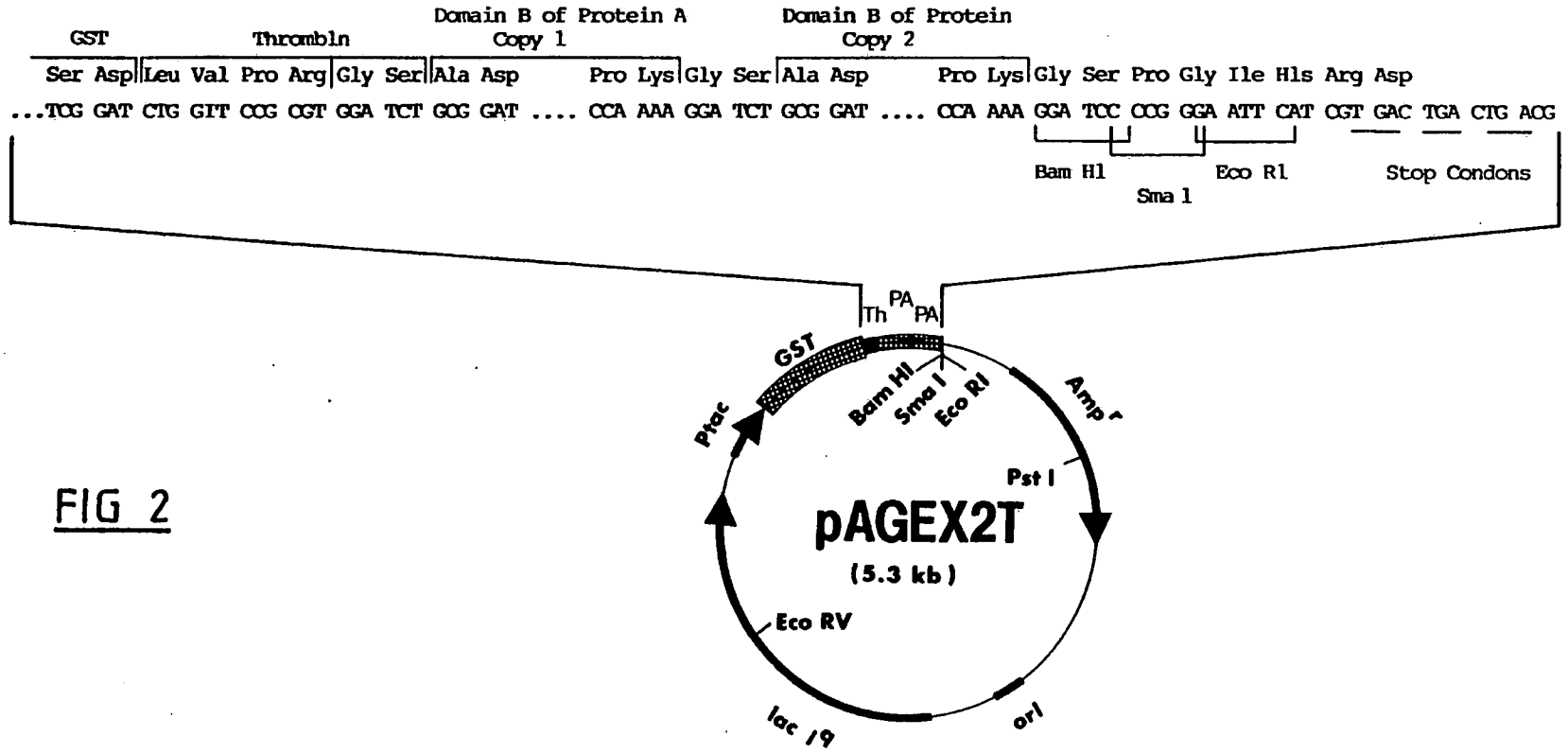

FIG 2

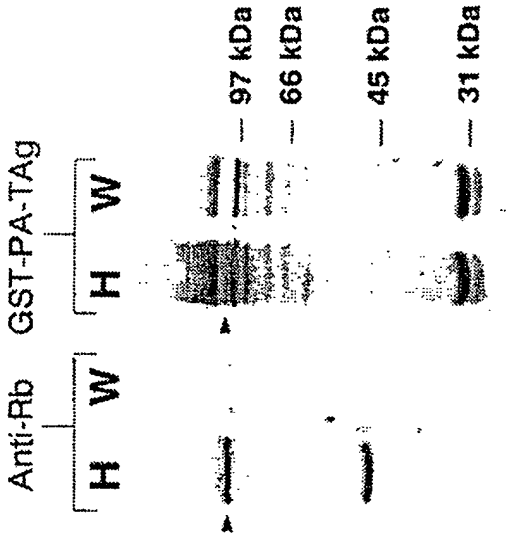

FIG 5

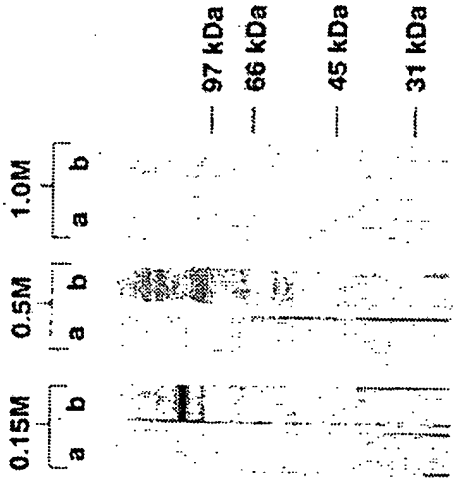

FIG 4

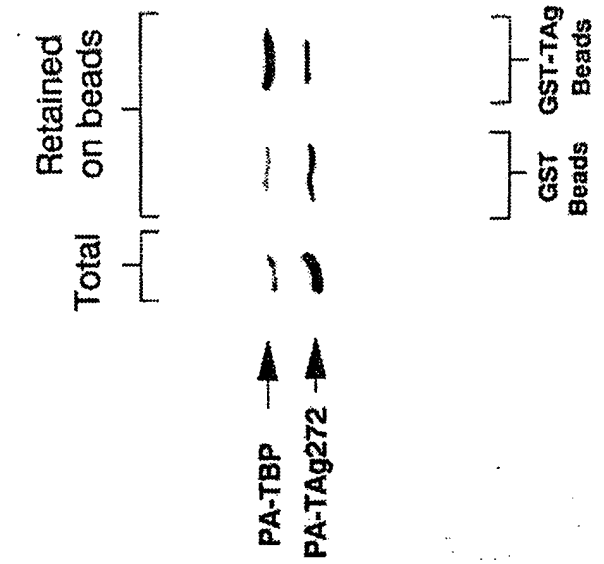

FIG 7

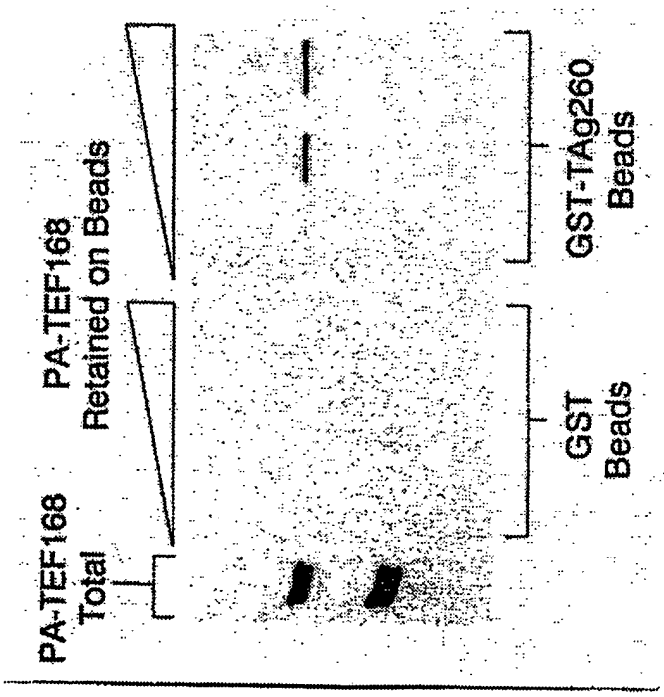

FIG 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU93/00105

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] C07K 15/00,15/12, C12N 9/10,15/00,15/54,15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC AS ABOVE
Chem Abs. online KEYWORDS: Glutathione S-transferase, Glutathione S-alkyl-transferase, Glutathione S-aryl-transferase, Glutathione S-aralkyl-transferase OR S-hydroxyalkyl-glutathione lyase and fusion.
WPAT

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,P	AU,A,12051/92 (AMRAD CORPORATION LIMITED) 6 August 1992 (06.08.92) see page 6 lines 5-21, example 1 and claims 1 and 18-20	22 and 25
A	AU,A,17932/88 (AMRAD CORPORATION LIMITED) 21 December 1988 (21.12.88) see claims in particular.	
A	Molecular and Biochemical Parasitology, Volume 27, issued 1987 September, (Elsevier Scientific Publishing Company, Netherlands), Smith D.B. et al "Expression of an enzymatically active parasite molecule in E. coli: S. japonicum glutathione S-transferase" see pages 249-256.	

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 April 1993 (30.04.93)	19 MAY 1993 (19.05.93)
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer A. BESTOW Telephone No. (06) 2832295

Form PCT/ISA/210 (continuation of first sheet (2)) (July 1992) copdcm

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation f document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
A	Gene, Volume 58, issued 1987 June (Elsevier Scientific Publishing Company, Netherlands) B. Lowenadler et al "A gene fusion system for generating antibodies against short peptides" see pages 87-97.	
A	Gene, Volume 67, issued 1988 March (Elsevier Scientific Publishing Company, Netherlands) D. Smith and S. Johnson "Single-step purification of polypeptides expressed in E. coli as fusions with glutathione S-transferase" see pages 31-40.	
A	Journal of Immunological methods, Volume 136, issued 1990 October (Elsevier Scientific Publishing Company, Netherlands) A. Lew, D. Beck and L. Thomas "Recombinant fusion proteins of protein A and protein G with glutathione S-transferase as reporter molecules" see pages 211-219.	

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
AU,A 12051/92	WO 9213000

END OF ANNEX